HOW DOES KERATIN 17 CONTROL GENE EXPRESSION?

by

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Abstract

The type I intermediate filament protein keratin 17 has been long implicated as a meaningful biomarker for a broad range of carcinomas (Moll et al., 1982), and there is emerging evidence that its expression impacts tumorigenesis and related processes. For instance, we recently showed that the genetic loss of K17 delays tumor onset in a mouse model of basal cell carcinoma, correlating with reduced tumor keratinocyte proliferation and a dramatic changes in the immune response in skin, from Th1/Th17- to a Th2-dominated cytokine profile (DePianto et al., 2010). There also is evidence that K17 impacts psoriasis, where again it would contribute to the dysregulation of the immune response (Fu and Wang, 2012). These and other studies suggest a link between K17, chronic hyperproliferative and inflammatory skin diseases, and the regulation of cytokine gene expression. The molecular mechanisms accounting for this intriguing link are not understood. A previously reported mass spectrometry-based screen (Chung et al., 2012) identified hnRNP K, an RNA/DNA-binding protein involved in many aspects of gene expression, as a K17 binding partner. Here we show that hnRNP K binds mRNA transcripts encoding the CXCR3 ligands CxCl9, CxCl10, and CxCl11, which are involved in pathogenesis of human basal cell carcinomas (Lo et al., 2010). Furthermore, induction of these genes is both K17- and hnRNP K-dependent, in both the A431 human skin cancer line and *Gli2*$^{tg/+}$ mouse keratinocytes in primary culture. We also show that the expression of the *CxCl9, CxCl10,* and *CxCl11* depends on RSK kinase activity. These findings identify a candidate mechanism through which hnRNP K and K17 exert a pro-tumorigenic influence, the robust stimulation of CXCR3 ligand expression.

Thesis Committee:

Dr. Pierre A. Coulombe and Dr. Fengyi Wan

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Table of Contents

List of Figures

Introduction

The metazoan cytoskeleton

Before the 1990s, it was mostly accepted that the cytoskeleton is a unique feature of eukaryotes, a view that since then has been overturned. Today, bacteria are known to posses actin- and tubulin-like proteins (de Boer et al., 1992; Bork et al., 1992) that are essential for cell division. This underlines the evolutionary importance of the cytoskeleton for reasons beyond a need for structural and mechanical support. Given the acquisition of various membrane organelles, eukaryotes needed means of addressing trafficking issues — the increased complexity of eukaryotic cells, therefore, was accompanied by a major expansion and diversification in cytoskeletal proteins (Wickstead and Gull, 2011).

Due to a lack of cell wall, metazoa face a significant challenge when it comes to providing mechanical support for their cells and tissues. Metazoans generally rely on two systems to provide rigidity and support for their tissues. Each cell possesses its own cytoskeletal system and cells are connected to one another and their environment in tissues using various types of adhesive contacts. The two entities, the cytoskeleton and the adhesion apparatus, are often integrated to give rise to integrated supracellular networks. Accordingly, the cytoskeleton participates in multiple dynamic processes enabling both mechanical motion within the cell and migration of the entire cell (reviewed in Herrmann et al., 2007).

Metazoan cytoskeletal proteins are commonly classified by the diameter of the filaments they form: the 25 nm microtubules, the 10 nm intermediate filaments and the 7 nm microfilaments. These filaments are then organized together to form an intricate cytoskeletal network, in part via the involvement of a family of proteins called plakins (see Fig. 1; Leung et al., 2001).

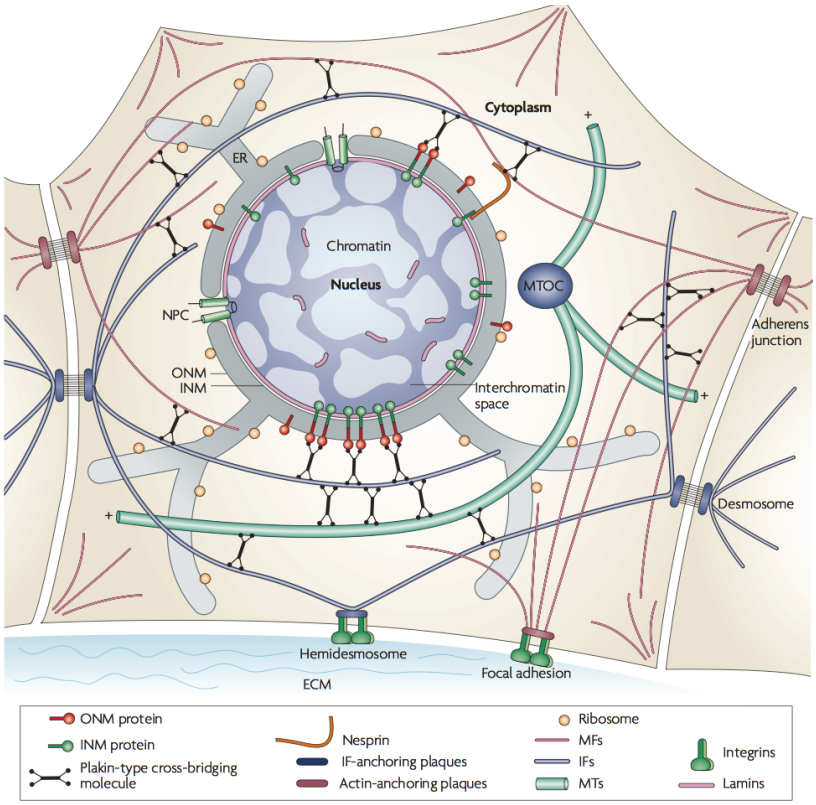

Figure 1. Organization of metazoan cytoskeleton
Microtubules (MTs), Intermediate filaments (IFs) and Microfilaments (MFs) combine with plakins to form metazoan cytoskeletal architecture. Cytoskeletons of neighboring cells are linked via different cell-to-cell junctions as well as anchored within the extracellular matrix (ECM). From Herrmann et al., 2007.

Cytoskeletal proteins connect neighboring cells to each other and to the extracellular matrix, creating a complex structural multicellular network that is crucially important for mechanotransduction (Katsumi et al., 2004; Shafrir and Forgacs, 2002) .

Among cytoskeletal proteins, intermediate filament proteins are the most diverse and heterogeneous group (Chung et al., 2013) and, in light of this thesis, deserve special attention.

Intermediate filaments

Despite their functional diversity, intermediate filament proteins share common structural features and can be broadly classified into six different subfamilies (Fig. 2A; Chung et al., 2013) based on gene substructure and sequence homology. About 70 different genes encoding for intermediate filaments exist in the human genome, with keratins accounting for

(a)

Subgrouping	Proteins	Cell type specificity
Type I	Keratins	Soft complex epithelia (skin, oral mucosa, etc.)
Type II		Soft simple epithelia (liver, gut, kidney, etc.)
		Hard epithelia (hair, nail, oral papillae)
Type III	Vimentin, Desmin	Various (fibroblasts, leukocytes, endothelium
	GFAP, Peripherin	muscle, astrocytes, glia, peripheral nerves)
	syncoilin	
Type IV	NF-L, NF-M, NF-H	CNS & neurons
	a-internexin	CNS & neurons
	synemin, nestin	Muscle, neural stem cells
Type V	Lamins A, B & C	Nucleus
Orphan	Filensin, Phakinin	Lens

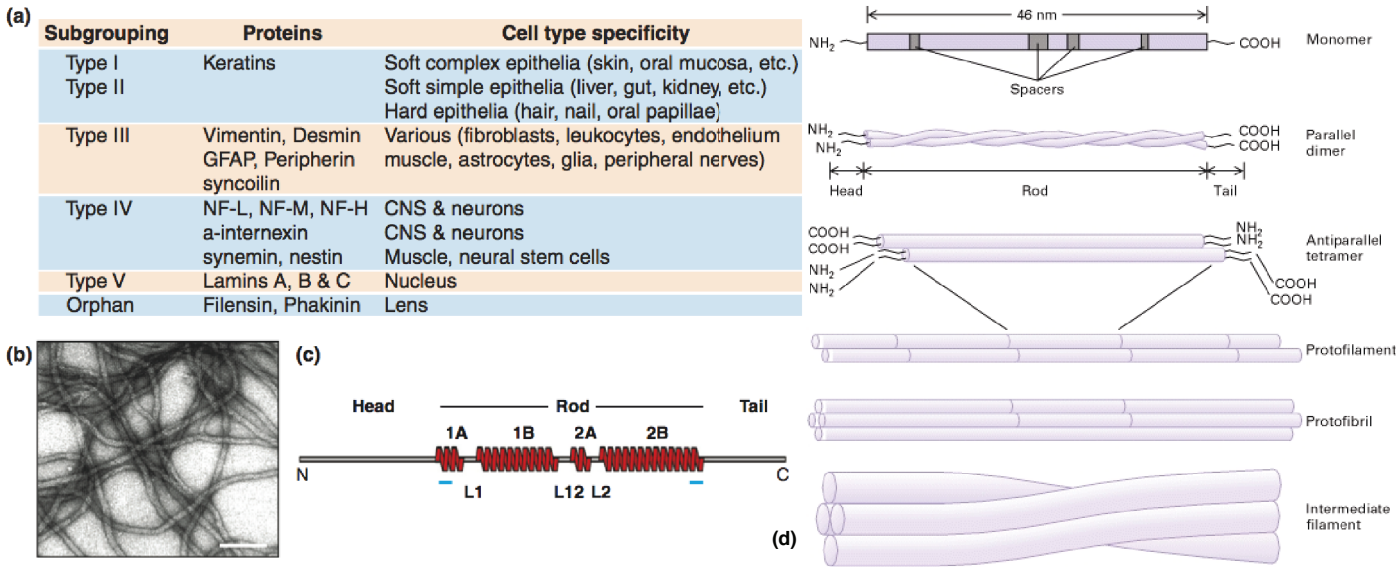

Figure 2. Diversity, structure and polymerization of intermediate filaments
IFs can be grouped into six groups based on their expression (a). (b) Electron microscopy image showing 10 nm keratin filaments. (c) Common structural organization shared by intermediate filament proteins. (a)-(c) from Chung et al., 2013, (d) from Lodish et al., 2000.

54 of these genes partitioned into two sequence types, the type I and type II intermediate filament proteins.

Though the molecular mass of intermediate filament proteins varies substantially (from 40 kDa to 240 kDa), they possess a conserved, centrally-located α-helical rod domain that is essential for 10 nm filament assembly. The rod domain is flanked by N- and C-terminal head and tail domains, respectively, which are highly variable and generally unstructured (Fig. 2C). Canonically, the majority of intermediate filament proteins are cytoplasmic, with the exception of the nuclear lamins. While cytoplasmic intermediate filaments are known to

impact nuclear cytoarchitecture (Lee et al., 2012), recent data from our laboratory and others indicates that keratins (a class of intermediate filament proteins) can also be found within the nucleus (Dr. Ryan Hobbs and Justin Jacob, unpublished data; Kumeta et al., 2013). Intermediate filament proteins first assemble to form a dimer, with the two monomers oriented in parallel and in register. Dimers then interact along their lateral surfaces with an anti-parallel orientation to form structurally apolar tetramers. Tetramers interact through lateral and end-to-end interactions to give rise to mature 10 nm filaments (see Fig. 2B) that exhibit no known structural polarity, a hallmark feature of microtubules (MTs) and microfilaments (MFs). Another distinguishing feature of IF proteins is that they do not bind nucleotides (e.g., ATP, GTP), which are essential to steady state dynamic properties of F-actin and microtubules. Intermediate filaments are implicated in a staggering variety of cellular functions, and, when perturbed, are responsible for a myriad of human diseases (see The Human Intermediate Filament Database, Szeverenyi et al., 2008). For the purposes of this review, we will focus on the keratin gene family.

Keratin gene family

Evolutionary, keratins are conserved in higher metazoans and mammals and, in humans, are represented by 54 functional genes. Out of these, 17 genes are encoding for keratins whose expression is largely restricted to hard epithelia (e.g., hair shaft, nail), while the remaining 37 are expressed in soft epithelial tissues. Keratin genes can be readily divided into two IF sequence types based on a phylogenetic approach. The protein products of types I and II pair with one another to form obligate heterodimers that polymerize to form mature keratin filaments. Type I keratin proteins are acidic in nature and are encoded by 28 genes in the human genome, while type II keratin proteins are basic to neutral and are encoded by the remaining 26 genes (Schweizer et al., 2006). Keratin proteins range from 40

kDa (type I K19) to 70 kDa (type II K1) in molecular mass: type II basic keratins usually have higher molecular mass (40-55 kDa for type I and 56-70 kDa for type II keratins; Moll et al., 2008).

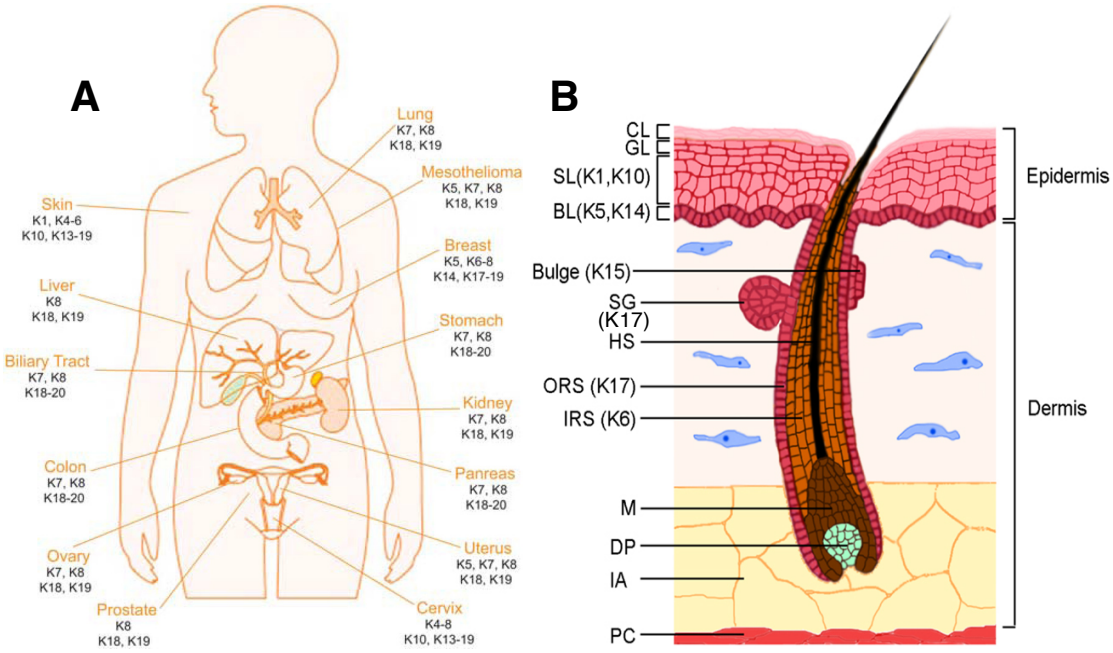

Figure 3. Specificity of keratin expression
(A) Differential expression of keratins throughout human organs and tissues. (B) Specificity of keratin expression in normal human skin layers. Notice, that as epidermal cells differentiate, K5/K14 expression is being phased out, while expression is K1/K10 is increasing. CL – cornified layer, GL – granular layer, SL – spinous layer, BL – basal layer, SG – sebaceous glands, HS – hair shaft, ORS, IRS – outer and inner root sheath, M – matrix, DP – dermal pappila, IA — intradermal apidocytes, PC — panniculus carnosus, a muscle layer supporting the skin. Images are from: (A) Karantza, 2011; (B) Antonini et al., 2013.

Different pairs of type I and type II keratins are differentially expressed in a tissue- and context-dependent fashion (Coulombe and Omary, 2002; Moll et al., 2008; Stoler et al., 1988), as well as in response to various stimuli (see Fig. 3; Antonini et al., 2013; Karantza, 2011).

Keratins perform a wide variety of functions in cells, which can broadly be divided into mechanical and non-mechanical roles (Kim and Coulombe, 2007; Toivola et al., 2010). The importance of keratin filament networks for cell and tissue mechanics is revealed when they are mutated in human disease, such as epidermolysis bullosa simplex (EBS) or epidermolytic

hyperkeratosis (EHK; see McLean and Moore, 2011 for a review). EBS arises when K5/K14 filaments are disturbed by mutations in their genes and manifests in extreme tissue fragility following exposure to minor mechanical forces, resulting in painful blisters. EHK is a form of ichtyosis caused by mutations in either K1 or K10, and presents with general blistering and hyperkeratosis. Since K1/K10 are mostly expressed in more differentiated cells of the epidermis, blisters in EBS are typically deeper and more painful. A key property of keratin filaments that contributes to their importance for cell and tissue mechanics is their connection to cell-to-cell and cell-to-matrix adhesive contacts, thereby forming a supracellular network contributing to overall tissue rigidity (reviewed in Simpson et al., 2011). Structural studies have also pointed out an important role for keratins forming a perinuclear cage that affects the size and shape of the nucleus (Lee et al., 2012). Nuclear geometry has been implicated in modulating gene expression (Chen et al., 1997).

Different keratins (among other IF proteins) are post-translationally modified, which is crucial for their participation in signal transduction pathways that allow for non-mechanical regulatory functions. Keratins are known to be phosphorylated, ubiquitinated, SUMOylated, glycosylated and acetylated at various sites (Snider and Omary, 2014) and these modifications contribute, in part, to modulation of their interactions with partner proteins. Non-mechanical functions of keratins regulate many aspects cell physiology: cell survival and death, embryonic development and tissue homeostasis, and cell growth and motility (Fig 4, see Chung et al., 2013; Pan et al., 2013 for a review).

Accumulating evidence suggests that keratins are actively involved in the modulation of immune responses, a phenomenon of special interest to this thesis. A number of keratins have demonstrated immunomodulatory properties. In mice, genetic ablation of *Krt8* causes colitis characterized by an increased Th2 profile associated with an inflammatory response (Habtezion et al., 2005). Genetic ablation of *Krt1* in mice is associated with an inflammatory

skin disease accompanied by the activation of the inflammasome and massive secretion of IL-18, a potent proinflammatory cytokine, by K1 null epidermal keratinocytes. Release of IL-18 was demonstrated to be keratinocyte-autonomous and depletion of IL-18 partially rescued the defect (Roth et al., 2012). Keratin 16 was demonstrated to play a role in innate immunity. Mice lacking *Krt16* exhibit phenotypic features that are clearly related to pachyonychia congenita (Lessard and Coulombe, 2012), a human disease that is caused by mutations in the *KRT16*, *KRT17* or the *KRT6* genes and is characterized by nail dystrophy, palmoplantar keratoderma (massive thickening and hyperkeratosis of the palms and soles), along with oral and glandular lesions. Additionally, loss of *Krt16* in mice induces levels of several damage-associated molecular pattern genes (DAMPs), which serve as effectors for innate immune responses (Lessard et al., 2013). Absence of Keratin 5, a gene responsible for EBS, induces classical Th1 markers *IL-1b* and *IL-6* in mouse skin and tissue culture models of EBS (Lu et al., 2007). Additionally, ablation of *Krt5,* but not *Krt14* in a mouse model of EBS, induced expression of Ccl2, Ccl19 and Ccl20, all of which are nF-kB-dependent genes and involved in recruitment of Langerhans cells (a resident dendritic cell in the skin). This finding was in agreement with data obtained from skin of EBS patients with *K5* mutations, but not *K14* (Roth et al., 2009).

Inflammation and immune responses are now widely regarded as drivers of tumorigenesis (Couzin-Frankel, 2013). Of special interest to us is Keratin 17 which, as demonstrated by DePianto et al. (2010), plays an important role in both immunomodulation and cancer.

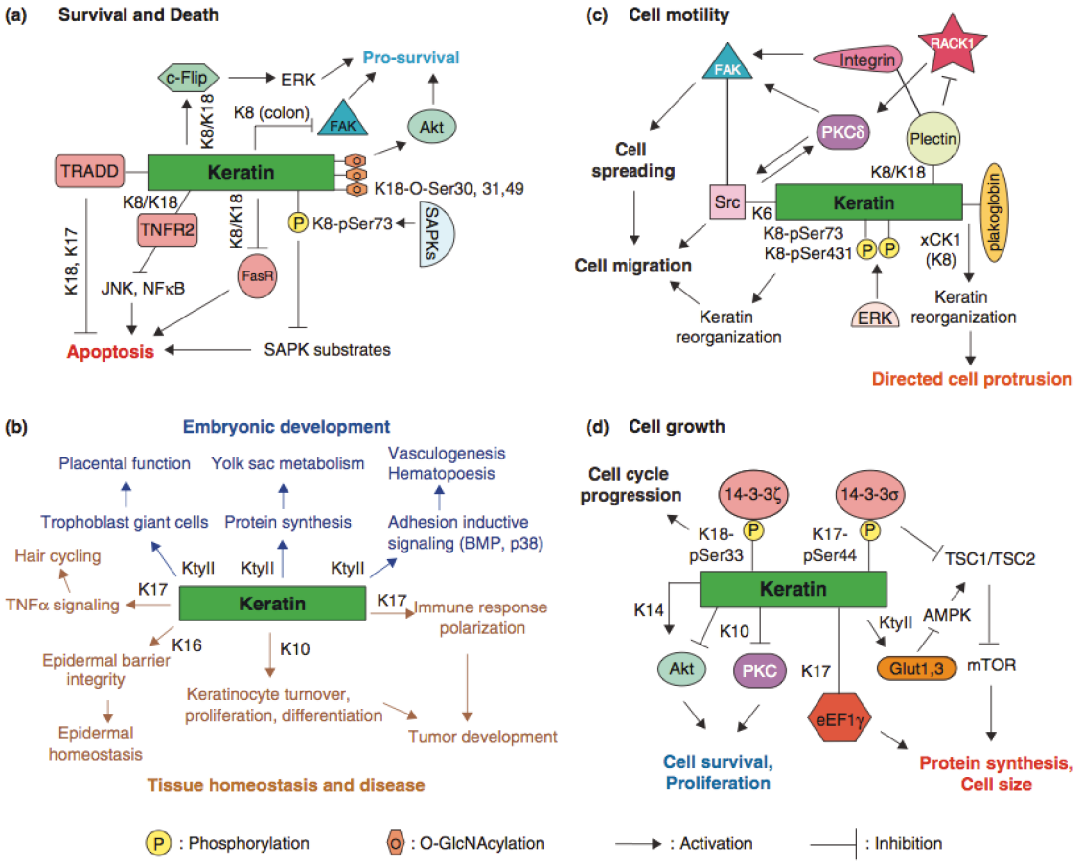

Figure 4. Non-mechanical functions of keratins
Different keratins regulate various pathways during development, growth, cell division, survival and homeostasis, cell motility and programmed cell death. Keratins accomplish these functions by interacting with a myriad of signaling proteins. Frequently, these interactions are mediated by elaborate post-translational modifications of keratins. Image from Pan et al., 2013.

Keratin 17: at the crossroads of immunity, inflammation and cancer

Keratin 17, a 46 kDa type I keratin, is often referred to as an inducible keratin. Keratin 17 expression in normal tissues is largely restricted to ectoderm-derived epithelial appendages, including hair follicles, nail beds, sebaceous glands and oral papilla. A great mouse model to visualize K17 expression pattern in the skin takes advantage of *K17* promoter to express GFP (Bianchi et al., 2005). In addition, K17 expression can be induced by a variety of stimuli, among which are wounding (McGowan and Coulombe, 1998), IFNγ (Vogel et al., 1995), EGF (Amit et al., 2007), IL-17A (Shi et al., 2011) and IL-22 (Zhang et al., 2012), all of which are typically prominent in the setting of an inflammatory response.

Mutations in *KRT17* cause human diseases, namely pachyonichia congenita type II (McLean et al., 1995) and a condition called steatocystoma multiplex (Covello et al., 1998), characterized by appearance of sebum-filled cysts in the skin.

Psoriasis is an example of a well-studied skin disease setting in which K17 is robustly induced (de Jong et al., 1991). Psoriasis is a chronic, inflammatory skin disorder characterized by hyperproliferation and aberrant differentiation of keratinocytes (Fu and Wang, 2012). While the exact cause of psoriasis is yet to be identified, dysregulation of keratinocytes and immune cells are key features of this condition (Lowes et al., 2007). Induction of K17 expression occurs very early during the development of psoriatic plaques and correlates with an immune shift in skin tissue towards a pro-inflammatory Th1/Th17 dominated response (Fu and Wang, 2012; Zheng et al., 2007). In addition, K17 peptides containing ALEEAN sequence may serve as an autoimmune epitope in psoriasis, promoting proliferation of T-lymphocytes and exaggerating the condition (Shen et al., 2005; see Gudmundsdottir et al., 1999). In light of these findings, K17 has been suggested as a potential candidate target for psoriasis therapy (Fu and Wang, 2012). In addition to having an emerging role in the pathogenesis of psoriasis, multiple lines of evidence link K17 to various cancers (Markey et al., 1992; Moll et al., 1982). Indeed, K17 is expressed in basal cell carcinoma (Markey et al., 1992), breast (Nielsen et al., 2004), cervical (Smedts et al., 1992), lung (Chen et al., 2011), pancreatic (Sarbia et al., 2007; Yang et al., 2012), gastric (Ide et al., 2012) and ovarian (Wang et al., 2013) cancer. K17 expression often correlates with poorer prognosis in these cancer patients (Figure 5).

Despite a significant amount of information about K17 as a marker for various cancers, the exact molecular function of K17 expression in these settings is yet to be defined. A number of studies have examined K17's roles in signaling cascades. K17 was found to control hair follicle cycling in a TNFα-dependent fashion (Tong and Coulombe, 2006), which is an interesting connection to a pro-apoptotic TNFα pathway. Another defined role of K17 is

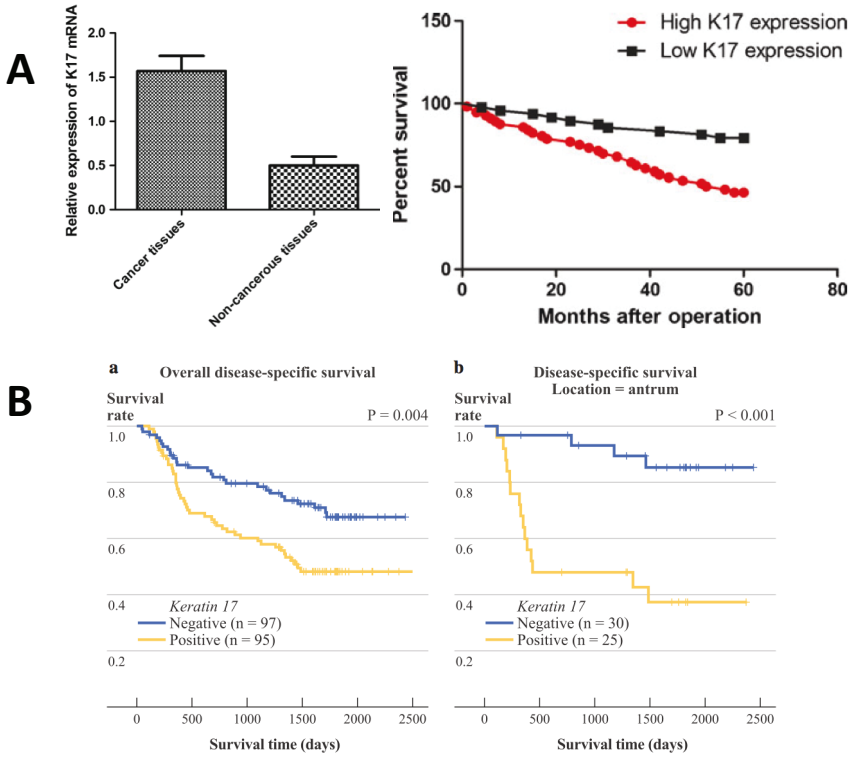

Figure 5. K17 expression in tumors correlates with poorer patient prognosis
In case of epithelial ovarian cancer (A), cancerous tissues express significantly more K17 (A, left panel), which correlated with decreased survival rate after operation (A, right panel; Wang et al., 2013). In case of gastric adenocarcinoma (B), K17-expressing tumors tend to be more aggressive, which is reflected by decreased both overall survival time (B, left panel), and is even more apparent if tumor was found in the antrum region (B, right panel; Ide et al. 2012)

to regulate protein synthesis and cell growth. Phosphorylated K17 is able to interact with 14-3-3σ, increasing cytoplasmic presence of this adaptor protein and leading to activation of the Akt/mTOR pathway, protein synthesis, and epithelial cell growth (Kim et al., 2006; Sankar et al., 2013). This set of findings is especially exciting in light of emerging data regarding the differential regulation of T-helper lymphocyte responses by mTORC1 and mTORC2

complexes (Delgoffe et al., 2011). K17 expression correlates with polarization of immune responses in psoriasis (see above) and additional evidence comes from a recent study by DePianto et. al. (2010). In this latter study, the impact of genetically ablating *Krt17* was examined in a mouse model of basal cell carcinoma, driven by sustained expression of a Gli2 transgene (*Gli2*$^{tg/+}$) in the skin. In this setting, the absence of K17 not only delayed tumor onset (Fig. 6), but also polarized immune profile from a Th1/Th17 to a Th2-dominated profile. The observed effect was K17-specific and did not occur when the *Krt14* gene was similarly ablated, despite 73% sequence identity between the K17 and K14 proteins.

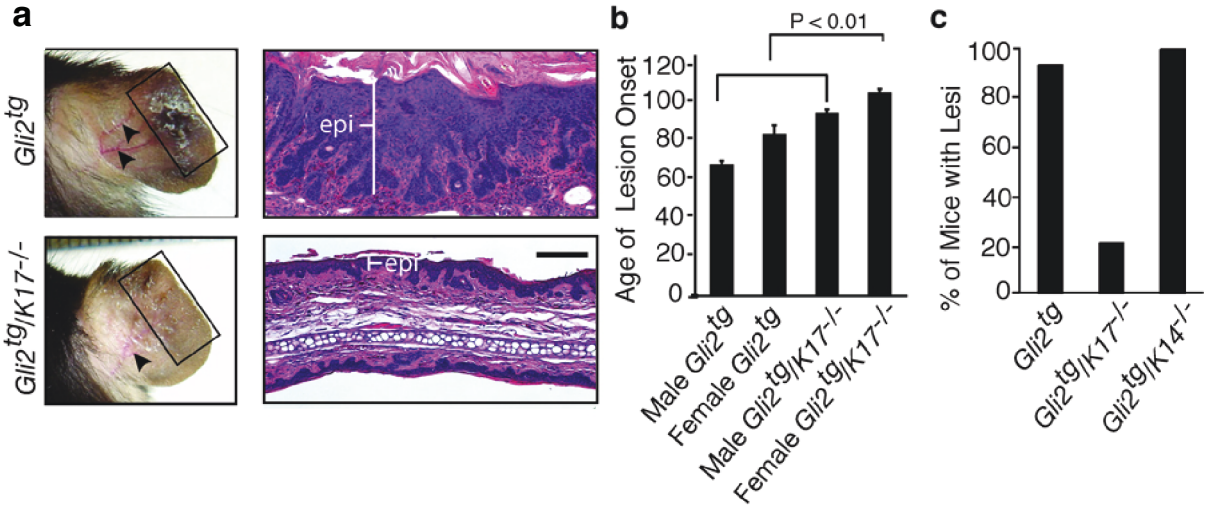

Figure 6. Absence of K17 delays tumor onset in mouse model of basal cell carcinoma.
(a) *Gli2*^{tg} mice spontaneously develop BCC-like tumors on their ears, and tumor onset is delayed when K17 null mice are used. Left panel shows a typical ear lesion at P80. Right panel shows dramatic thickening of the epithelium in the BCC-like tumor. Epithelial thickness is reduced when K17 is absent. (b) and (c) quantification of onset delay (b) and percentage of mice with tumors at P80 (c) caused by absence of K17. Image from DePianto et al., 2010.

The study by DePianto et al. thus provided important clues about a possible molecular mechanism by which K17 may exhibit tumor-promoting properties and laid a solid foundation for deeper investigation of this phenomenon. To address this, a mass spectrometry-based screen for K17-binding proteins was conducted in our laboratory (Chung et al., 2012). Analysis of such K17-associated partners identified heterogenous nuclear ribonucleoprotein K (hnRNP K), a member of hnRNP family of proteins that are involved in various processes

related to gene expression (Han et al., 2010). The K17-hnRNP K interaction was further confirmed by reciprocal endogenous coimmunoprecipitation assays (Dr. Byung Min Chung, unpublished data), thus paving the way for an investigation of the functional significance of this interaction.

hnRNP K and tumorigenesis

hnRNPs are a family of multifunctional proteins with a central role in the regulation of mRNA maturation, splicing, DNA damage repair, telomere maintenance and other functions (Chaudhury et al., 2010; Makeyev and Liebhaber, 2002). hnRNPs can post-transcriptionally regulate expression of mRNAs by binding to 3'-UTR regions (Swanson and Dreyfuss, 1988) and participate in the regulation of RNA stability. hnRNPs are organized in RNP particles — multimolecular protein/RNA complexes of variable composition that are thought to be nucleated by hnRNP C (König et al., 2010) protein. There is a subset of hnRNP proteins, termed poly(C)-binding proteins, that show a preference for binding to poly(C)-rich sequences on RNAs (PCBPs; Dreyfuss et al., 1993). The 51 kDa hnRNP K protein is a member of this subset (Matunis et al., 1992).

The amino acid sequence of hnRNP K shows three distinct "K domains" discovered to represent a common RNA-binding motif, now defined as "K-homology (KH) domains" (reviewed in Valverde et al., 2008). The hnRNP K protein also possesses a nuclear localization signal (NLS; Michael et al., 1997) and, therefore, is predominantly nuclear in normal tissues. This said, it has been observed in the cytoplasm, in mitochondria, and in the vicinity of the plasma membrane (Mikula et al., 2006). hnRNP K is also known to be phosphorylated by various kinases that affect its function (Habelhah et al., 2001a; Ostareck-Lederer et al., 2002; Sataranatarajan et al., 2008). For example, hnRNP K phosphorylation by ERK promotes its cytoplasmic accumulation and has been associated with inhibition of

translation of hnRNP K-dependent mRNAs (Habelhah et al., 2001b). hnRNP K can also participate in signaling cascades by recruiting ERK components directly to sites of transcription (Mikula and Bomsztyk, 2011)

hnRNP K is a particularly interesting ribonucleoprotein, in part due to the breadth of its interactome. hnRNP K interacts with a staggering number of proteins that perform a wide array of cellular functions (Mikula et al., 2006) and same can be said for the known RNA targets of hnRNP K (Liu and Szaro, 2011).

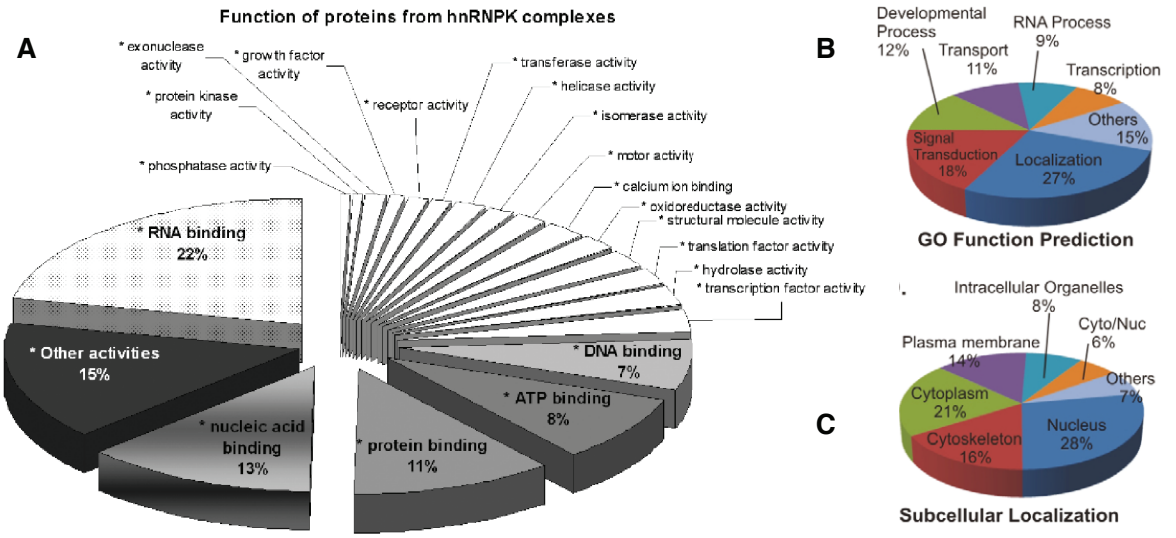

Figure 7. hnRNP K interactome
(A) Protein interactome of hnRNP K revealed interaction with proteins participating in various functions, the most common being RNA/nucleic acid/ATP binding. Mikula et al., 2006. hnRNP K-bound RNAs participate in many cellular functions as well (B) and are localized to various sites within the cell (C). Liu and Szaro, 2011.

hnRNP K has already been implicated in inflammation and cancer progression. For instance, the importance of hnRNP K in inflammation is manifested through its ability to control the stability of COX2 mRNA (Shanmugam et al., 2008), which encodes an enzyme crucial for synthesis of prostaglandins. The clinical importance of COX2 in inflammation has made this enzyme one the most common targets of non-steroidal anti-inflammatory drugs

(NSAIDs). In the cancer setting, hnRNP K is known to be overexpressed, enhancing cellular proliferation and transformation (Barboro et al., 2009; Carpenter et al., 2006; Gao et al., 2013; Mandal et al., 2001; Roychoudhury and Chaudhuri, 2007) and, interestingly, the subcellular localization of hnRNP K becomes more cytoplasmic in tumors (Chen et al., 2009; Inoue et al., 2007). Given that our laboratory has discovered that the cytoplasmic localization of hnRNP K depends in part on K17 protein (Dr. Byung Min Chung, unpublished data), we reasoned that the impact of K17 on BCC tumor progression could be mediated, at least partly, by interaction with hnRNP K.

We hypothesized that K17 exhibits immunomodulatory effects on cytokine expression through its interaction with hnRNP K, identified by Dr. Byung Min Chung. Therefore, we decided to test association of hnRNP K with K17-dependent targets (using the RIP assay) encoding for these cytokines and investigate their dependence on hnRNP K by modulating hnRNP K expression.

Results

hnRNP K protein binds several mRNAs coding for potent cytokines

To better understand the role of hnRNP K in K17-mediated regulation of immune profile of cytokine expression, we first performed hnRNP K RNA immunoprecipitation (RIP) assays, which involves precipitation of target RNA-binding protein and subsequent isolation of bound RNAs. The procedure was performed using lysates obtained from both A431 human epidermoid carcinoma cells, which are known to express K17 among other keratins (Moll et al., 1982), and *Gli*$^{tg/+}$ mouse epidermal keratinocytes in primary culture. Isolated RNA from hnRNP K precipitates and negative control IgG was analyzed by qRT-PCR using target-specific primer pairs (see Materials and Methods). Consistently with published data, the mRNA transcripts coding for *MYC* (Evans et al., 2003), *PTGS2* (Shanmugam et al., 2008) *and EGR1* (Mikula and Bomsztyk, 2011) are highly enriched in hnRNP K RIPs in both types of cell lysates, relative to negative control IP (Fig 8). mRNAs coding for various chemokines and cytokines, including *IL4, IL17C, AIRE* and TSLP, are also selectively enriched in hnRNP K precipitates. An interesting observation is that the *KRT17* transcript also precipitates effectively in the hnRNP K RIP assay, raising the prospect that K17 expression might be by hnRNP K at the transcriptional or posttranscriptional level. Of special interest to us are the mRNAs encoding *CXCL9*, *CXCL10* and *CXCL11*, the protein products of which are ligands for the CXCR3 receptor. These ligands (and their cognate receptor) function to regulate immune cell trafficking during Th1 immune responses, which is promoted by K17 in the *Gli2*^{tg} mouse model of basal cell carcinoma (DePianto et al., 2010). The CXCR3 axis has well established functions during tumor development in various models (Kawada et al., 2007; Lo et al., 2010; Ma et al., 2009; Wu et al., 2012). Expression of the *CXCL9*, *CXCL10* and

CXCL11 genes was found to be robustly K17-dependent, in three different experimental paradigms exploited in our laboratory:the *HPV*^{tg} mouse, the *Gli*$^{tg/+}$ mouse and human A431 cells treated with 12-O-tetradecanoylphorbol-13-acetate (TPA; Dr. Ryan Hobbs, unpublished data), a phorbol ester which induces contact dermatitis (Hickey et al., 1981) with expression of various cytokines via the NF-kB pathway (Catley et al., 2004).

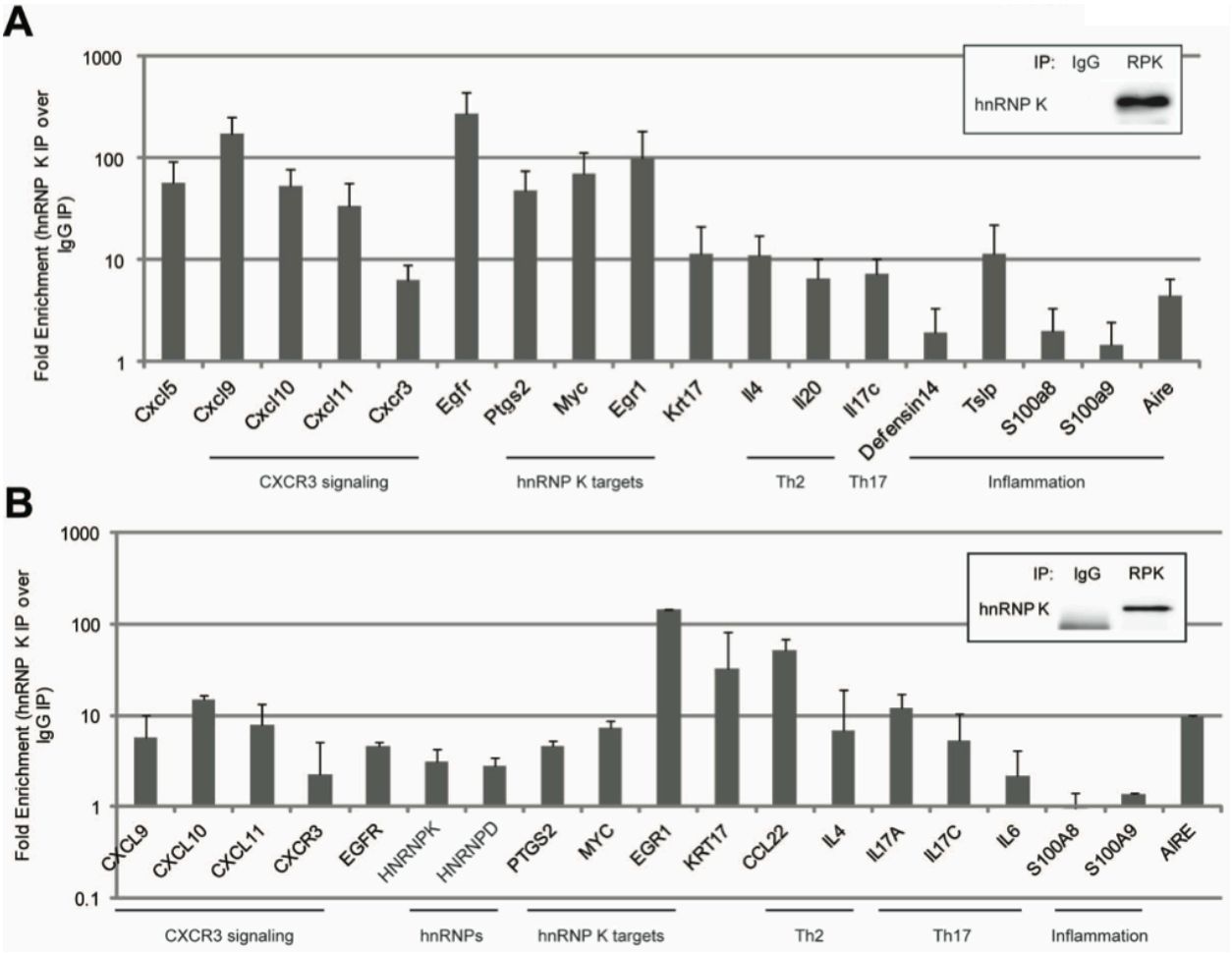

Figure 8. hnRNP K RNA immunoprecipitation assays.
hnRNP K RIP assays were performed using primary keratinocytes from newborn *Gli2*^{tg} *Krt17*$^{+/-}$ mice (A) or A431 cells (B) and analyzed by qRT-PCR. Data is normalized to IgG control. Insets demonstrate successful hnRNP K immunoprecipitation relative to normal IgG negative control as shown by anti-hnRNPK immunoblotting of precipitates. Error bars represent SEM. A log scale is used for the Y-axis (fold-enrichment).

To test whether the association of hnRNP K with *CXCL9*, *CXCL10* and *CXCL11* mRNAs requires K17, we performed RIP assays using A431 cells stably expressing either a *KRT17*-directed shRNA or a scrambled control (see Chung et al., JBC 2012). Cells were

serum-starved prior to stimulation with TPA (or DMSO vehicle control) and RIP was performed (Fig 9 C). Additionally, RIP was performed using *Gli2*$^{tg/+}$ *Krt17*$^{-/-}$ keratinocytes (Fig. 9).

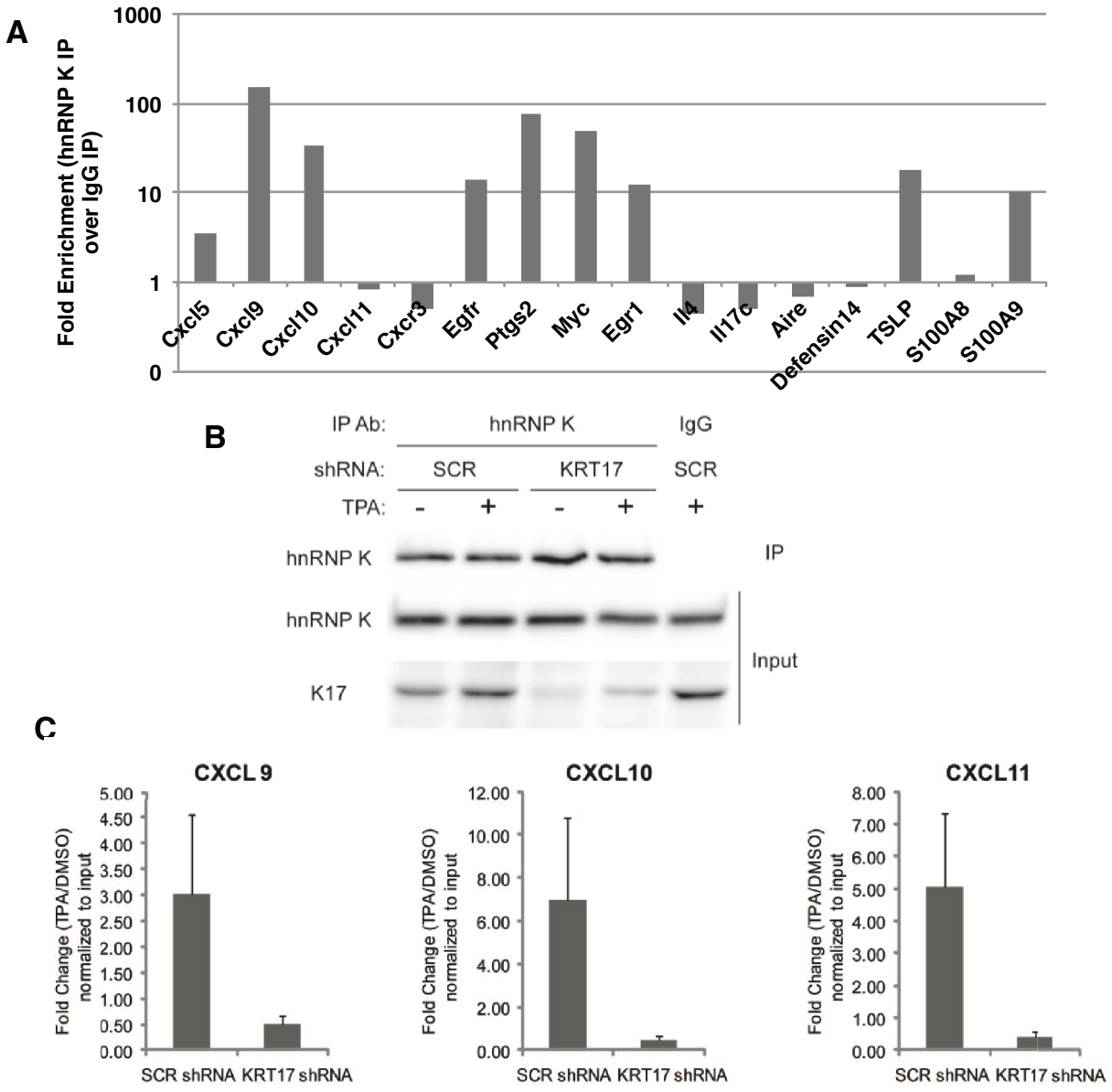

Figure 9. K17 enhances association of hnRNP K with selected transcripts
hnRNP K RIP assays were performed using primary keratinocytes from newborn *Gli*$^{t/+g}$ *Krt17*$^{-/-}$ mice. Data normalized to IgG control. (A) or A431 cells treated with TPA or DMSO vehicle control (C), and analyzed by qRT-PCR. For (C), data was normalized to DMSO-treated hnRNP K samples and inputs. In (B), anti-hnRNP K and anti-K17 antibodies were used to analyze immune precipitates (IP) or the input sample (Input) by immunoblotting. Where present, error bars indicate SEM. A log scale used for the Y-axis in (A).

LIkewise, complete absence of K17 (genetic null, Fig. 9 A) reduces association of hnRNP K with target transcripts in *Gli*$^{tg/+}$ mouse epidermal keratinocytes in primary culture, with the most dramatic decreases observed for *CXCL11* and *EGFR* transcripts. In contrast, association of hnRNP K with its previously known targets *(PTGS2, MYC, EGR1)* does not seem to be affected by the K17 status. The latter experiment needs to be repeated to ensure full reproducibility. When RIP is carried out using serum starved A431 cells treated with TPA or DMSO, a stronger association between hnRNP K protein and the *CXCL9*, *CXCL10* and *CXCL11* transcripts occurs upon TPA treatment in cells stably expressing scrambled shRNA compared to cells expressing KRT17 shRNA. These findings establish hnRNP K protein as a binding partner and a potential regulator for a significant number of cytokine mRNAs, in a K17-dependent fashion, and also strongly suggests hnRNP K's involvement in CXCR3 signaling cascade, which has important implications for carcinogenesis.

CXCL9, CXCL10 **and** ***CXCL11*** **transcripts are hnRNP K-dependent**

To examine the role of hnRNP K in the regulation of the *CXCL9, CXCL10* and *CXCL11* transcripts, we carried out siRNA-mediated knockdown of hnRNP K in A431 cells, and assessed whether TPA-induced upregulation of these mRNAs occurs normally. All three hnRNP K siRNAs tested, designed to target different regions of *HNRNPK*, demonstrated high knockdown efficiency (96%, 83% and 91%, for siRNA1, siRNA2 and siRNA3 at the mRNA level, respectively; see Fig 10 A). siRNA1 was used in all subsequent experiments. As expected, compared to non-silencing (NS) control siRNA, hnRNP K knockdown A431 cells showed a dramatic decrease in their ability to induce cytokine mRNAs (namely *CXCL9*, *CXCL10* and *CXCL11*) following TPA treatment (Fig 10 B). The *CXCR3* mRNA does not behave in the same way, nor does *IL4*, which is an important Th2 marker, supporting the importance and specificity of hnRNP K-K17 interaction in promoting a Th1, rather than a Th2,

response. An interesting observation is that the *KRT17* mRNA behaves similarly to *CXCL9*, *CXCL10* and *CXCL11,* which is in agreement with the hnRNP K RIP results whereby the *KRT17* transcript is robustly enriched compared to negative control. The full significance of

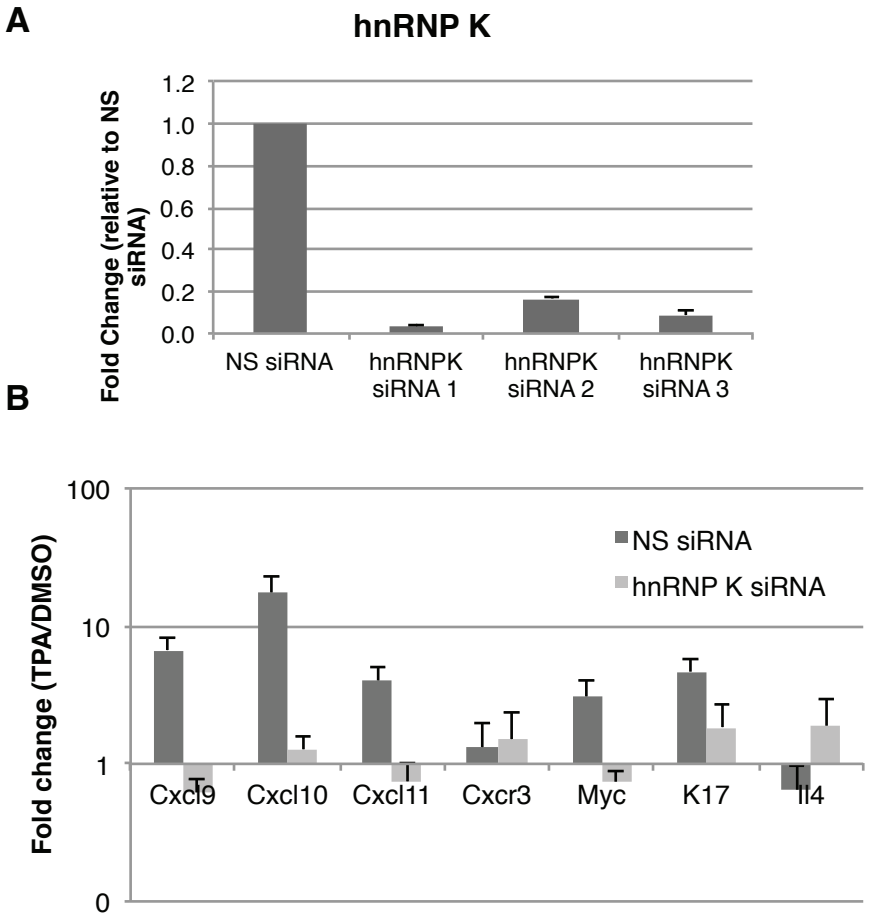

Figure 10. hnRNP K siRNA knockdown in cultured A431 tumor keratinocytes.
hnRNP K was knocked down using siRNAs targeting different regions of *HNRNPK* (A), all of which demonstrated great efficiency in decreasing steady state *HNRNPK* levels, with siRNA 1 being the most effective. (B) A431 cells transfected with non-silencing (NS) or hnRNP K siRNA1 were serum starved and treated with TPA. RNA was isolated and analyzed by qRT-PCR. Error bars represent SEM.

the latter finding remains to be established, through additional experimentation. Taken together with the RIP-based results, the outcome of these experiments indicates that not only does hnRNP K bind to *CXCL9*, *CXCL10* and *CXCL11*, but also regulates their expression, at least in the TPA paradigm.

hnRNP K cooperates with K17 to regulate expression of CXCR3 ligands

To test whether hnRNP K depends on K17 to mediate CXCR3 ligands expression, we next overexpressed mCherryC1-hnRNPK in A431 cells. Transfecting A431 cells is known to be challenging, with high cytotoxicity being an issue, and we therefore decided to first test a variety of transfection reagents (Lipofectamine3000, FugeneHD, Fugene6, GeneJuice, Xfect, Continuum, and TransIT) with the mCherryC1-vector (Fig 11 A). Gross cell morphology, along with upregulation of target genes in response to TPA, served as complementary reporters for relative cytotoxicity. All the reagents tested decreased the responsiveness of A431 cells to TPA. FugeneHD (FuHD) was selected for our studies based on its combination of relatively low toxicity and acceptable retention of TPA responsiveness. Next, mCherryC1 Vector control or mCherryC1-hnRNPK were overexpressed in A431 cells to test whether hnRNP K overexpression increases the response to TPA stimulation, relative to vector control (Fig. 11 B). hnRNP K was successfully overexpressed as evident from (Fig. 11 D, left), and cells expressing hnRNP K were indeed found to be more responsive to TPA-induced upregulation of CXCR3 ligands (Fig. 11 B). As with the hnRNP K knockdown, mRNA levels for the Th2 marker IL4 were not affected in this experiment (Fig. 11 B). To investigate whether K17 plays a role in hnRNP K's effects on CXCR3 ligand transcript levels, we next overexpressed hnRNP K in either KRT17 shRNA-expressing A431 cells or A431 cells expressing control shRNA (Fig11 C; for degree of overexpression is demonstrated in Fig. 11 D, right). A431 cells expressing control shRNA exhibited higher TPA responsiveness upon hnRNP K overexpression compared to cells expressing KRT17 shRNA, as showed by higher relative levels of *CXCL9, CXCL10* and *CXCL11* mRNAs. Such a cooperation between hnRNP K and K17 is especially interesting in light of recent findings, from in our laboratory, showing that K17 affects the subcellular localization of hnRNP K (Dr. Byung Min Chung, unpublished data), together with the observation that presence of hnRNP K in the cytoplasm

is important in carcinogenesis (Lundberg et al., 2010; Uhlén et al., 2005; Zhou et al., 2010). Additionally, K17 was recently found to occur within the nucleus (Dr. Ryan Hobbs,

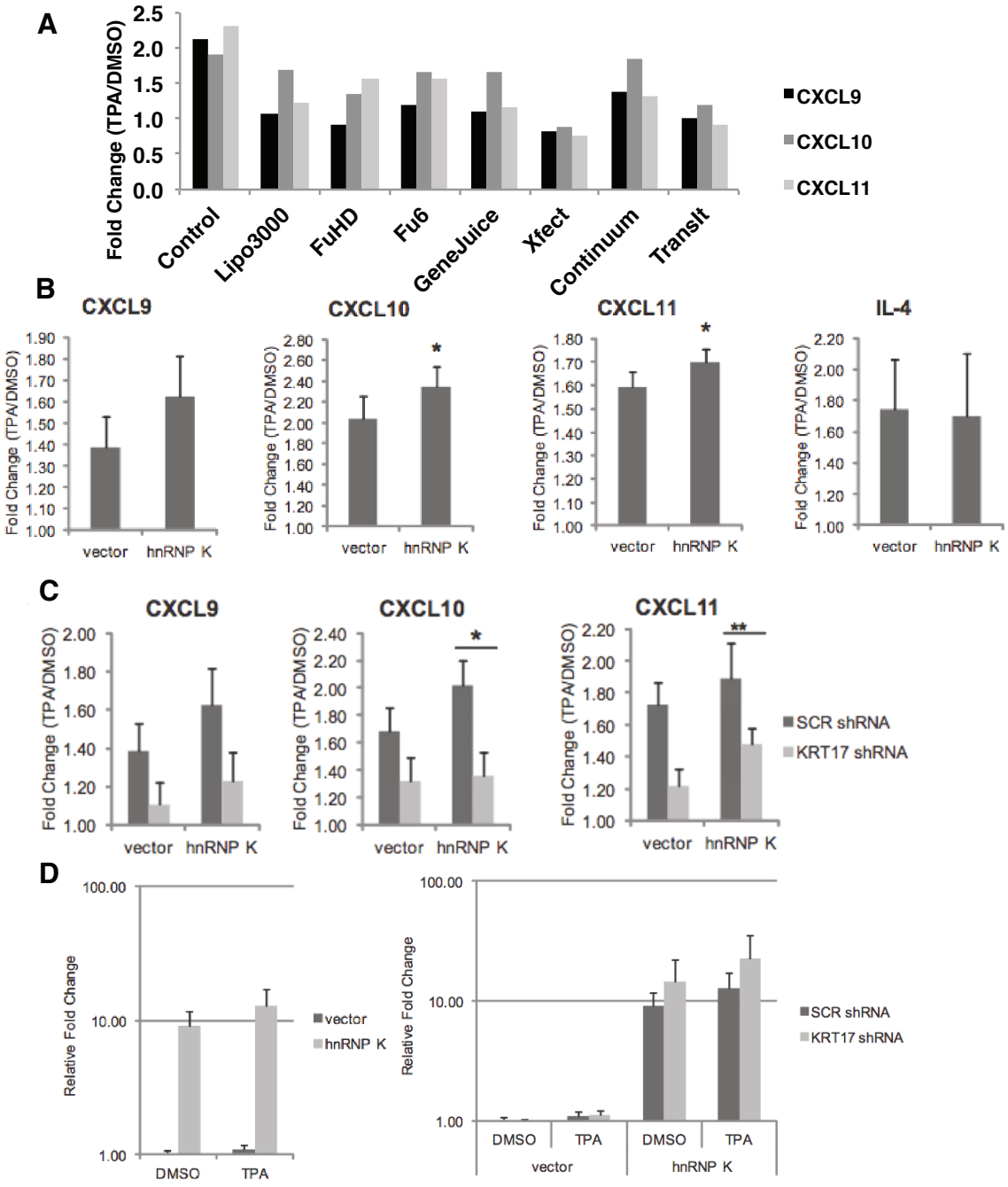

Figure 11. hnRNP K regulates CXCR3 ligands expression in K17-dependent fashion
(A) Comparison of different transfection reagents to assess their effects on the response of A431 cells to TPA treatment. (B,C) mCherryC1-hnRNP K was overexpressed in A431 cells (B) or A431 cells stably expressing KRT17 shRNA or scrambled control (C). Cells were serum starved and treated with TPA, and RNA was isolated and subjected to qRT-PCR analysis. Frame (D) shows efficiency of hnRNP K overexpression using FuGene HD reagent in A431 cells (left panel) and in SCR or KRT 17 shRNA-treated A431 cells (right panel).

unpublished data), opening exciting possibilities for a broad and far ranging relationship between hnRNP K and K17, and dynamics of this interaction in response to various stimuli. Taken together, these findings establish that expression of CXCR3 ligands is both hnRNP K- and K17-dependent.

K17 impacts the stability of CXCR3 ligand mRNAs

hnRNP K participates in formation of various RNP complexes that have defined roles in RNA metabolism such as splicing and RNA degradation (Bomsztyk et al., 2004). To test whether K17 exhibits any effect on RNA stability (that can be possibly mediated by K17-hnRNP K interaction), we carried out an "actinomycin D pulse chase experiment". Actinomycin D is a potent transcription inhibitor and allows for the monitoring of mRNA decay over time. To test for the stability of CXCR3 ligand mRNAs, SCR shRNA- or KRT17 shRNA-expressing A431 cells were serum-starved and pretreated with DMSO or TPA for 1.5 hours to induce transcription of CXCR3 ligands. After TPA treatment, cells were incubated with actinomycin D or DMSO control for 4 hours. We found that the *CXCL9, CXCL10* and *CXCL11* mRNA levels are similar between actinomycin D- and DMSO-treated cells, indicating that these transcripts are relatively stable in cells when K17 levels are normal, at the 4 hours time point (Fig. 12). However, cells expressing KRT17 shRNA showed decreased levels of *CXCL11* mRNA in actinomycin D-treated cells compared to those treated with DMSO, with a weaker impact on *CXCL9* and *CXCL10* mRNAs. This experimental outcome suggests that the effects of K17 on the levels of CXCR3 ligand mRNAs are, at least partly, due to a difference in RNA stability. More experiments are needed to confirm this interpretation (see Discussion).

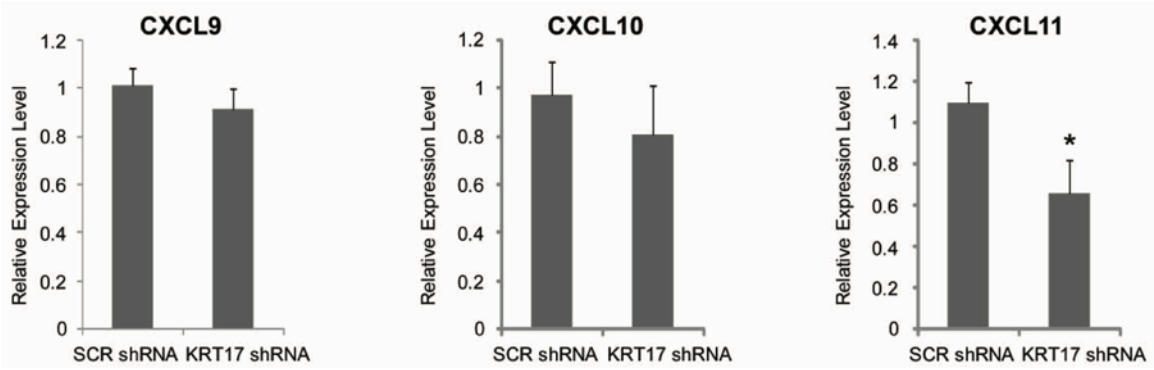

Figure 12. Use of Actinomycin D to test the stability of specific mRNAs.
Serum starved A431 cells expressing scrambled (SCR) or KRT17 shRNA were initially treated with TPA and subsequently with actinomycin D for 4 hours. RNA was isolated from cells and analyzed by qRT-PCR. Bars indicate fraction of initial RNA remaining in actinomycin D-treated cells compared to DMSO-treated cells.

RSK is involved in regulating the expression of CXCR3 ligands

Both K17 and hnRNP K are phosphorylated by various kinases. hnRNP K is modified by c-Src, JNK, PKC and Erk kinases (Habelhah et al., 2001a; Ostareck-Lederer et al., 2002; Sataranatarajan et al., 2008) and physically interacts with RSK (Kanlaya et al., 2010), while K17 is known to be phosphorylated by RSK kinase (Pan et al., 2011). With so many different kinases possibly involved in regulation of the K17-hnRNP K interaction, all of which are potent mediators of signal transduction pathways, we decided to use a panel of specific inhibitors to see if inhibition of any kinase will show a pronounced effect on induction of CXCR3 ligand expression (Fig. 13 A).

RSK inhibition led to the strongest attenuation of the response of A431 tumor keratinocytes to TPA. p38 inhibition also decreased CXCR3 ligands induction. PKA and PKC inhibitors exhibited little effect, while, interestingly, MEK and ERK inhibition resulted in increased TPA responsiveness due to considerably lowered steady state levels of CXCR3 ligand mRNAs. We decided to focus our subsequent efforts on RSK and performed siRNA-mediated knockdown of RSK in A431 cells to test whether the outcome observed will mimic the effect of inhibiting the enzyme.

As expected, the outcome of a RSK knockdown closely resembled that of RSK inhibition, strongly attenuating CXCR3 ligands induction upon TPA stimulation (Fig. 14). Decrease in TPA response seems to be more pronounced with lower levels of K17 in *KRT17* shRNA expressing cells. More experiments are necessary, however, to establish statistical significance. This data strongly suggests that RSK activity is necessary for induction of CXCR3 ligands.

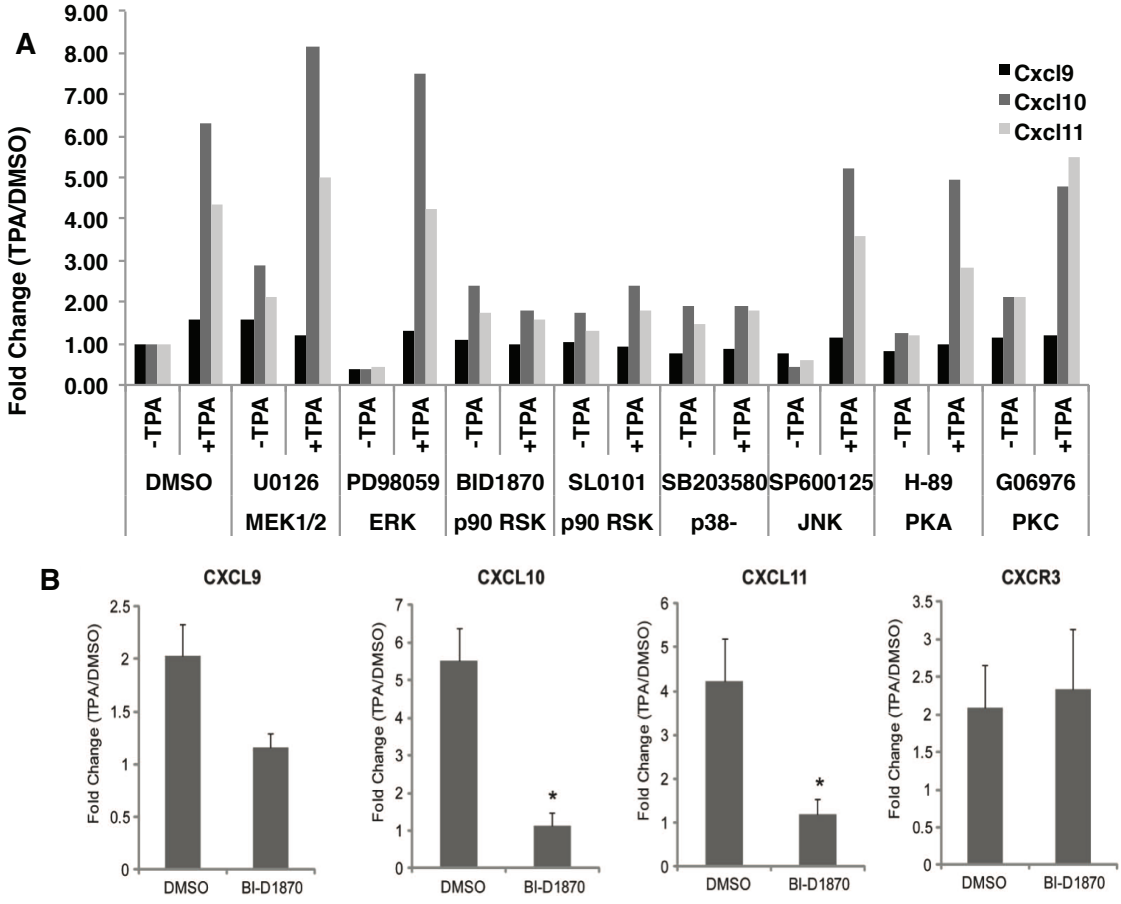

Figure 13. Kinase inhibition suggests that RSK involvement in regulating CXCR3 ligands
(A) A431 cells were pre-treated with various kinase inhibitors or DMSO control and subsequently stimulated with TPA (+TPA) or DMSO (-TPA). RNA was harvested and analyzed by qRT-PCR. X axis labels indicate names of inhibitors and their target kinases. Data is normalized to DMSO. (B) Pretreatment of A431 cells with BI-D1870, a potent RSK inhibitor, followed by TPA treatment and qRT-PCR analysis revealed that RSK activity is necessary for proper induction of CXCR3 ligands mRNAs, but not the receptor mRNA. Error bars indicate SEM.

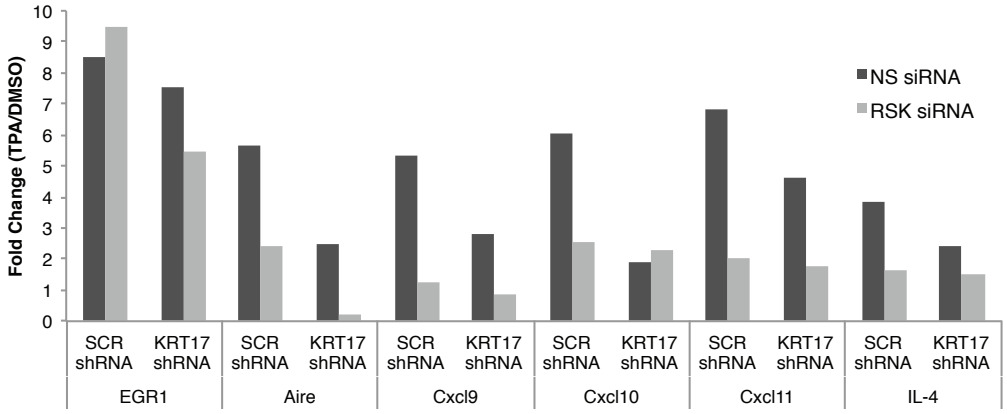

Figure 14. RSK siRNA-mediated knockdown
SCR shRNA or KRT17 shRNA-expressing A431 cells were transfected with NS or RSK siRNA and treated with TPA. RNA was harvested and analyzed by qRT-PCR. Data normalized to DMSO control.

Different stimuli exhibit different degrees of K17-dependence

In addition to TPA, which induces CXCR3 expression in a K17-dependent fashion (Fig. 15 A), we decided to test additional stimuli to induce CXCR3 ligands. SCR or KRK17 shRNA expressing A431 cells were treated with EGF or IFNγ, which are known inducers of K17 expression. Both treatments robustly induced CXCR3 ligands expression, however, these responses did not exhibit K17 dependence, as in the case for TPA (Fig 15). Since some degree of K17-dependence was observed at lower concentrations of IFNγ, more experiments are needed to fully understand response to these stimuli with regards to K17 status.

These findings underline that K17's contribution in regulating the immune response *in vivo* is likely to be very specific. Even more importantly, with many cell types and signaling cascades involved in establishment of Th1 adaptive response, interplay and balance between K17-dependent and K17-independent responses becomes complicated and needs to be examined carefully.

Together, these results suggest existence of a K17-hnRNP K-CXCR3 axis, that is likely important for the observed effects of K17 on tumorigenesis *in vivo*.

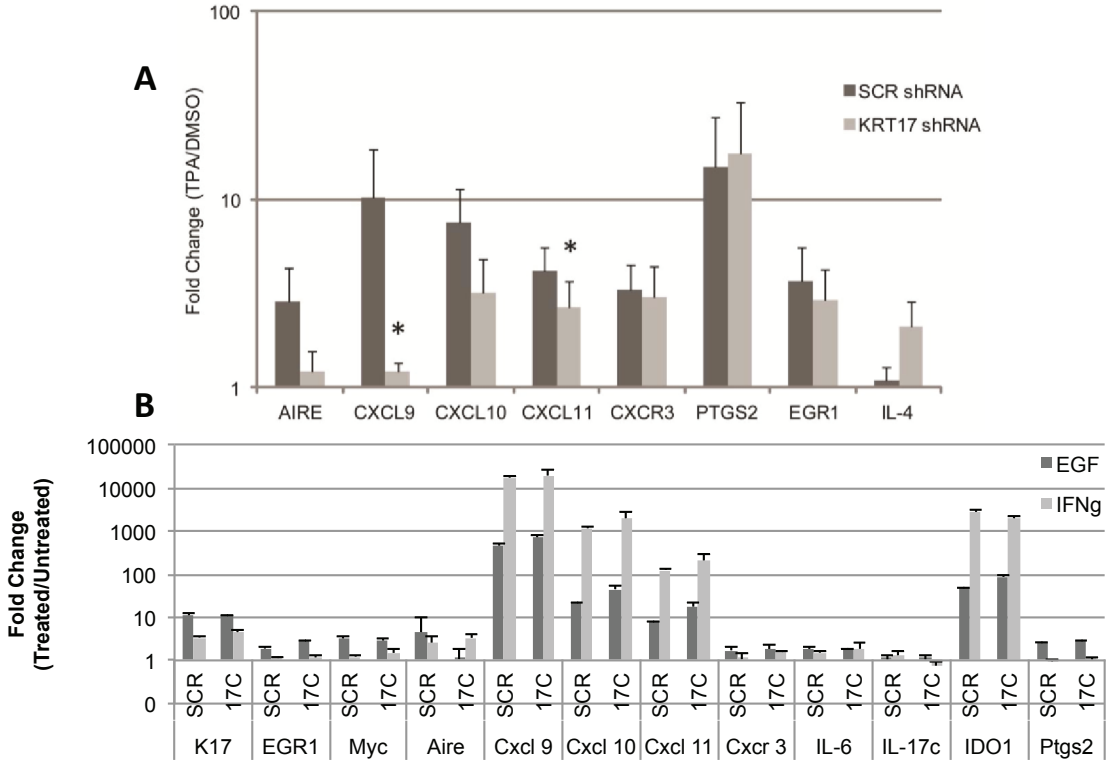

Figure 15. Treatment with different stimuli
SCR shRNA or KRT17 shRNA-expressing (17C) A431 cells were serum starved and treated with TPA (A) or (B) EGF or IFNγ RNA was harvested and analyzed by qRT-PCR. Log scale used. Error bars represent SEM.

Discussion

Proper appreciation of the findings reported in this thesis call for the consideration in the broader setting of the hnRNP K project in the Coulombe laboratory and, in particular, the substantive data set generated by Dr. Byung Min Chung. In Dr. Chungs' experiments, K17 was found to be important for cell transformation in an anchorage-independent cell growth assay, as well as cell invasion in a Matrigel invasion assay. These effects were found to be mediated by CXCR3 signaling, and elevated levels of CXCL11 and CXCL9 proteins were observed by ELISA in the growth medium containing cells with normal K17 level compared to cells expressing K17 shRNA. Further, using anti-CXCR3 antibody or a chemical antagonist successfully prevents K17-dependent cell transformation as assessed in the anchorage-independent growth assay. Therefore, cells expressing normal levels of K17 are secreting pro-inflammatory molecules that are responsible for the observed pro-inflammatory properties of K17 *in vivo.* Indeed, substantially more immune cells infiltrate the tumorigenic ear lesions occurring in *Krt17*$^{+/+}$*;Gli2*$^{tg/+}$ than in *Krt17*$^{-/-}$*;Gli2*$^{tg/+}$ animals (DePianto et al., 2010). hnRNP K was also found to be implicated in this process: siRNA-mediated knockdown of hnRNP K decreases the number of transformed colonies in anchorage-independent growth assay along with lowers levels of CXCL11 in the serum. The cytoplasmic level of hnRNP K was also decreased in ear lesions of *Krt17*$^{-/-}$*;Gli2*$^{tg/+}$ mice compared to those in *Krt17*$^{+/+}$*;Gli2*$^{tg/+}$ mice (Dr. Byung Min Chung, unpublished data), thus supporting the involvement of hnRNP K in K17-dependent tumorigenesis. However, more studies are required to define the exact role of hnRNP K in *Gli2*$^{tg/+}$ mice. Therefore, multiple lines of evidence suggest that K17 and hnRNP K partner up to regulate inflammation and tumorigenesis via, at least partially, CXCR3 pathway. This is especially intriguing considering that each of the key players identified in

these experiments has been individually implicated in the carcinogenesis and inflammation in various models. The novelty of these observations resides in the fact that K17, hnRNP K and CXCR3 signaling are found to work closely together to contribute to the observed phenotype. Furthermore, this study highlights importance of RSK, another prominent player in cancer development (Anjum and Blenis, 2008; Sulzmaier and Ramos, 2013), in regulating effect of the K17-hnRNP K interaction and its downstream effects.

A number of questions follow from this body of work and need to be addressed in the future to foster a better understanding of the emerging K17-hnRNPK-CXCR3 signaling axis, some of which will be discussed here, especially in light of recent findings in our laboratory.

A pressing issue is to define the binding interface between hnRNP K and K17. Understanding which regions of the K17 and hnRNP K proteins are particularly important for their interaction may provide useful tools to further understand the physiological significance of this interaction, and a promising target for intervention that may be of clinical benefit. To address this issue hnRNP K truncation mutants, or, on K17's side, various K17-K14 chimeras can be useful to investigate importance of various segments in each participating protein. Investigating the role of the posttranslational modifications known to occur on both proteins in regulating the K17-hnRNP K interaction also represents an interesting direction for future research.

From hnRNP K perspective, it might be of value finding out which region of the protein mediates K17-dependent effects on gene expression. hnRNP K may preferentially use any of its three KH domains for regulating K17-dependent target RNAs. Utilizing a non-biased analysis of hnRNP K RIPs, such as RIP-Seq, CLIP (crosslinking and immunoprecipitation) or iCLIP (individual-nucleotide resolution CLIP) (König et al., 2010), may help identify additional targets of hnRNP K that may also be K17-dependent. An additional benefit of using CLIP or iCLIP is the ability to identify hnRNP K-binding sites on the target bound RNAs.

While keratins are not known to bind DNA directly, our laboratory has observed the occurrence of K17 within the nucleus (Dr. Ryan Hobbs and Justin Jacob, unpublished data). Chromatin immunoprecipitation (CHIP) assays using anti-K17 antibody revealed an enrichment for select promoter sequences, among which are promoters for CXCR3 ligand-encoding genes. While this approach does not answer whether or not K17 itself directly binds DNA or this interaction occurs via another protein, an attractive (and conservative) possibility is the physical and functional involvement of K17 in a multi-protein complex in the nucleus. We are planning to examine this and related scenarios through the use of electrophoretic mobility shift assays (EMSAs) using antibodies for specific proteins of interest, and probes corresponding to relevant promoter sequences. A protein of particular interest is Autoimmune Regulator (AIRE), a transcriptional regulator that also interacts with K17 protein, both physically and functionally. The *AIRE* transcript is enriched in hnRNP K RIPs (this thesis), is expressed in mouse skin, and overexpression of AIRE protein restores the inability of K17 shRNA-expressing A431 cells to induce cytokines upon TPA stimulus (Dr. Ryan Hobbs, unpublished data). Given the hnRNP K RIP data, AIRE likely lies downstream of the K17-hnRNP K interaction. The impact of AIRE on the K17-hnRNP K interaction, and the associated downstream effects, are certainly of great interest but remains to be elucidated.

Another significant issue left unaddressed is discerning between a mode of action at the transcriptional vs. posttranscriptional effects for the K17-hnRNP K protein complex. Considering K17's presence in the nucleus, K17 may be regulating gene expression at both steps: for instance, from providing a scaffold for transcriptional regulatory complexes in the nucleus to influencing RNA stability through hnRNP K in the cytoplasm (the latter has been observed in this study; see Fig. 12). hnRNP K itself may also function at both the transcriptional and posttranscriptional levels —this remarkable protein is for instance known to be a part of the basal transcriptional machinery via interaction with TATA-binding protein

(TBP; Michelotti et al., 1996) and also regulate various aspects of RNA metabolism by binding target RNAs. Canonical hnRNP K-controlled transcripts (*EGR1, MYC, PTGS2*) all possess a poly-(C) motif, which are not readily obvious in the CXCR3 ligands mRNAs. Therefore, it is important to elucidate the hnRNP K binding sites on these mRNAs, and this may give additional clues as to how hnRNP K controls these transcripts as well as provide basis for a sequence homology search of other hnRNP K-regulated transcripts.

Another interesting observation is whether hnRNP K can regulate K17 itself. The *KRT17* transcript is robustly enriched in hnRNP K RIPs, and when hnRNP K siRNA is used, the expected increase in K17 expression following TPA treatment (as measured by RNA levels, and of a modest nature) does not occur as it does in control cells. At this point, this data remains inconclusive and more experiments need to be performed.

Combining the experiments reported in this thesis with those performed by Dr. Byung Min Chung lead us to propose a model whereby the RSK kinas-regulated interaction between K17 and hnRNP K interaction controls the expression of CXC3 ligand, as well as CXCR3-dependent signaling, in the setting of tumorigenesis. These finding are the first mechanistic attempt to explain how keratin 17, a cytoskeletal protein, can control gene expression in the cancer setting.

Materials and methods

Mouse models

C57BL/6 *Krt17*$^{-/-}$ mice were crossed to C57BL/6 *Gli2*$^{tg/+}$ transgenic mice to create *Krt17*$^{-/-}$*; Gli2*$^{tg/+}$ mice, and relevant controls, as described (DePianto et al. 2010). Genotyping was performed for the *Krt17* locus and *Gli2*$^{tg/+}$ transgene using PCR as per (DePianto et al. 2010).

Tissue culture

Human A431 cell lines (ATCC) stably expressing K17 shRNA or K17 scrambled RNA (Chung et al., 2012) were grown in 1g/L DMEM medium with glutamine (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin mixture (Gibco) and puromycin (Sigma). Cells were grown at 37°C with 5% CO_2. Subconfluent cultures were split by trypsinization (0.05% Trypsin-EDTA, Gibco), with trypsin deactivation by addition of 10% FBS DMEM. Cells were pelleted by centrifugation for 5 minutes at 4°C and 350 g. Pellets were resuspended in 10% FBS DMEM and plated on 10 or 20 cm dishes or 6-well plates (Corning). Primary cultures of skin keratinocytes from 2-day-old mouse pups of age-matched or littermate *Krt17*$^{-/-}$*;Gli2*$^{tg/+}$ and *Krt17*$^{+/+}$*;Gli2*$^{tg/+}$ mice were isolated as described (Bernot et al., 2004) and maintained in Cnt-57 media (CELLnTEC).

hnRNP K Overexpression

A431 cells were grown on 6-well plates until 50-60% confluent, and medium was changed to fresh 10% FBS DMEM. The *HNRNPK* cDNA was cloned out of pCMV6-AC hnRNP K (Origene) using PCR, with oligonucleotide primers (forward) 5′-

TCGAATTCTGATGGAAACTGAACAGCCAGAAG-3′ (EcoRI restriction site underlined) and 5′-ATCCCGGGCTTAGAATCCTTCAACATCTGCAT AC-3′ (reverse) (XmaI restriction site underlined) and cloned into mCherry-C1 (Clontech). 1.5 μg of plasmid DNA (pmCherry C-1 vector encoding mCherry-hnRNP K or vector control encoding mCherry) was transfected into each well by mixing it with Fugene HD reagent (Promega) using 3:1 ratio (3 μL of Fugene HD to 1 μg of DNA) in 200 μl of serum-free medium (OPTI-MEM, Gibco). Following 15 min incubation at RT, suspension was added to the cells dropwise. Next day, the medium was changed, and cells were lysed to harvest RNA at 48 hrs after transfection. Overexpression of HNRNPK was confirmed by qRT-PCR or Western blotting.

hnRNP K or RSK knockdown by RNA interference

A431 cells were grown on 6-well plates until 50-60% confluent, and medium was changed to fresh 10% FBS DMEM. hnRNP K knockdown was performed using designed duplex siRNA or scrambled siRNA as a control. The sequences used are as follow:

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hnRNP K siRNA:    5′-CCUAUGAAGCAGAGGAAUGUUGGCUUU-3′
                  5′-AGCCAACAUUCCUCUGCUUCAUAGG-3′
scrambled siRNA: 5′-GUCUAACAGCCGUUACACACCUUAGAU-3′
                  5′-CUAAGGUGUGUAACGGCUGUUAGAC-3′
```

“On target plus SMARTpool RSK1 siRNAs” from Dharmacon were previously described in a published report from our laboratory (Pan et al. 2011). 7μl of 10 μM Duplex RNA was mixed with 7μl Lipofectamine RNAiMAX (Invitrogen) in serum-free media (OPTI-MEM, Gibco). Following 5 min incubation at RT, suspension was added to the growing cells in wells of a 6-well plate dropwise. RNA was harvested after 48 hrs after transfection. Knockdown was accessed by qRT-PCR.

RNA isolation

Growing cells were washed twice with nuclease-free PBS (Ambion). RNA was isolated from cells using Clontech NucleoSpin RNA kit according to manufacturer's protocol. Concentration and purity of RNA samples (A260/230 and A260/280 ratios) was measured by spectrophotometry (IMPLEN P330 spectrophotometer).

cDNA synthesis and qRT-PCR

1 μg of RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions.

qRT-PCR was performed on 50 ng of cDNA per well (Hard Shell 96 well PCR plates, Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad) on C1000 Thermal Cycler (Bio-Rad) with CFX96 Real-Time system (Bio-Rad) using following reaction protocol:

1. 95°C for 5 min
2. 40 cycles of amplification (95°C for 10 sec; 60°C for 30 sec; repeat)
3. 95°C for 10 sec
4. Melting curve was plotted for 65°C-95°C in 0.5°C increments for 5 sec.

Amplification curves were visualized by CFX Manager 3.1 software (Bio-Rad). Relative fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method, normalizing to RPS18 and Actin levels. Percent input method was used to calculate fold enrichment as compared to the negative IgG control for RIP assays.

Target-specific primer pairs were synthesized by IDT and were as follows:

Gene	Direction	Primer sequence 5'- 3'
KRT17	Forward	GGTGGGTGGTGAGATCAATGT
	Reverse	CGCGGTTCAGTTCCTCTGTC
AIRE	Forward	CCAGGCTCTCAACTGAAGGC

	Reverse	GAATCCCGTTCCCGAGTGG
CXCL9	Forward	GTAGTGAGAAAGGGTCGCTGT
	Reverse	AGGGCTTGGGGCAAATTGTT
CXCL10	Forward	GTGGCATTCAAGGAGTACCTC
	Reverse	TGATGGCCTTCGATTCTGGATT
CXCL11	Forward	GACGCTGTCTTTGCATAGGC
	Reverse	GGATTTAGGCATCGTTGTCCTTT
CXCR3	Forward	CCACCTAGCTGTAGCAGACAC
	Reverse	AGGGCTCCTGCGTAGAAGTT
IL17A	Forward	TCCCACGAAATCCAGGATGC
	Reverse	TGTTCAGGTTGACCATCACAGT
IL6	Forward	AATTCGGTACATCCTCGACGG
	Reverse	TTGGAAGGTTCAGGTTGTTTTCT
IL4	Forward	CGGCAACTTTGTCCACGGA
	Reverse	TCTGTTACGGTCAACTCGGTG
S100A8	Forward	ATGCCGTCTACAGGGATGAC
	Reverse	ACACTCGGTCTCTAGCAATTTCT
S100A9	Forward	GGTCATAGAACACATCATGGAGG
	Reverse	GGCCTGGCTTATGGTGGTG
HNRNPK	Forward	GCAGGAGGAATTATTGGGGTC
	Reverse	TGCACTCTACAACCCTATCGG
HNRNPD	Forward	GCGTGGGTTCTGCTTTATTACC
	Reverse	TTGCTGATATTGTTCCTTCGACA
MYC	Forward	GGCTCCTGGCAAAAGGTCA
	Reverse	AGTTGTGCTGATGTGTGGAGA
PTGS2	Forward	CCAGTATAAGTGCGATTGTACCC
	Reverse	TCAAAAATTCCGGTGTTGAGCA
EGR1	Forward	GGTCAGTGGCCTAGTGAGC
	Reverse	GTGCCGCTGAGTAAATGGGA
IL17C	Forward	CCACACTGCTACTCGGCTG
	Reverse	CACACGGTATCTCCAGGGTGA
EGFR	Forward	AGGCACGAGTAACAAGCTCAC
	Reverse	ATGAGGACATAACCAGCCACC
ACTB	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAATA
RPS18	Forward	GCGGCGGAAAATAGCCTTTG
	Reverse	GATCACACGTTCCACCTCATC

Immunoprecipitation of hnRNP K-containing RNP complexes (RIP assay)

Cells were grown on 15 cm plates until 80-90% confluent. RIP assay was performed using RiboCluster Profiler RIP-Assay Kit (MBL Intl, Code RN1001) and hnRNP K pAb (MBL Intl, RN019P) according to manufacturer's protocol, with alterations. 30 ug of hnRNP K Ab or IgG control was used per confluent 15 cm plate, and antibodies were preincubated with 30 ul of Thermo Scientific Pierce Protein A/G Magnetic beads (Cat no. 88803) overnight. All the centrifugations were substituted for separations on a magnetic stand. After cells lysis in accordance with the MBL (manufacturer) protocol, equal aliquots of the lysate (input; 5% of total lysate;) were taken for quality control by Western blotting and RNA isolation followed by qRT-PCR. Quality control samples were also taken after incubating cell lysates (IP; 5% of total lysate;) with the immobilized antibody to confirm immunoprecipitation of hnRNP K. For qPCR analysis, as input was 5% of the IP, all data were normalized signals from the input reaction.

Testing of transfection reagents

A431 cells grown on 6-well plates were transiently transfected with Lipofectamine3000, FugeneHD, Fugene6, GeneJuice, Xfect, Continuum, and TransIT. The following amounts of reagents and DNA were used for the transfection:

Reagent (transfection time)	***OPTIMEM (ul)***	***Reagent (ul)***	***DNA (ug)***
1. No transfection control			
2. Lipofectamine 3000 (O/N) P3000)	250+250	3.75 (L3000)	2.5 (+ 5ul of
3. GeneJuice (O/N)	100	4.5	1.5
4. Continuum (O/N)	250	3.75	1.25
5. Xfect Clonetech (O/N)	100	7.5	2.5

6.	TransIT (O/N)	250	7.5	2.5
7.	Fugene6 (O/N)	100	4.5	1.5
8.	FugeneHD (O/N)	100	4.5	1.5

TPA stimulation and RNA harvest was performed 48 hrs after transfection.

Chemical treatments

Cells were serum starved in 0.1% FBS DMEM medium with puromycin for > 12 hours. TPA (12-O-Tetradecanoylphorbol-13-Acetate) was used at a 200 nM final concentration and treatments were done for 90 minutes prior to processing for RNA isolation. IFNγ was used at a 50 ng/mL final concentration for 6 hours. EGF was used at 25 ng/mL, also for 6 hours. Actinomycin D was used at 1 μg/mL concentration. Appropriate vehicle controls were used for each bioactive chemical tested (DMSO for TPA and Actinomycin D, PBS for IFNγ, 0.1% FBS DMEM for EGF).

When treated with RSK inhibitors, subconfluent and serum-starved A431 cells were first pretreated with an inhibitor (at 2.5 uM concentration) or DMSO vehicle control for 2 hours and subsequently treated with TPA for 1.5 hours.

Western blotting

Quality control samples from RIP assays were mixed 1:1 with 2X Laemmli sample buffer supplemented with 10% β-mercaptoethanol and boiled for 10 minutes. Samples were resolved by SDS-PAGE in 10% gels (Bio-Rad Power Pac 1000 source, Bio-Rad Casting Stand, Bio-Rad Mini Trans-Blot Cell tank) and transferred to 0.45μm nitrocellulose membrane (Bio-Rad) for 100 minutes at 100V. Membranes were then blocked with 5% bovine serum albumin (BSA, Sigma) in TBS-T buffer (w/w) for 1 hr at RT followed by an overnight incubation with primary antibodies against hnRNP K (3C2, Santa Cruz Biotechnology,

1:2000) or K17 (in house rabbit pAb, 1:10000) diluted in 5% BSA in TBS-T at 4°C. Next day, membranes were washed 3 times for 5 minutes with TBS-T and then incubated for 1 hr with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse for hnRNP K and anti-rabbit for K17) diluted 1:10000 in 5% BSA in TBS-T. Membranes then were washed again, before addition of GE Healthcare ECL Western Blotting Substrates. Chemiluminescence was detected using Alpha Innotech FluorChem Q imaging system.

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EDUCATION

2012-present: **Johns Hopkins Bloomberg School of Public Health**,
Department of Biochemistry and Molecular Biology (Baltimore, MD, U.S.A.).
Sc. M. candidate, Dr. Pierre Coulombe's lab.
First year (M.H.S.) thesis project: Impact of molecular chaperones on neurodegenerative disorders. Supervisor: Professor Roger McMacken, Ph. D.
Second year (Sc. M.) thesis project: How does Keratin 17 control gene expression?
Supervisor: Professor Pierre Coulombe, Ph. D.

2007-2012: **M. V. Lomonosov Moscow State University** (Moscow, Russia).
Department of Bioengineering and Bioinformatics
B.S./M.S. program
Fifth year (M.S.) thesis project: Autoubiquitination of an Agrobacterial Protein.
Supervisor: Professor Andrey Vartapetian, Ph.D., Dr. Sc.

1998-2007: School 1420 (Moscow, Russia)
Received "Silver medal" Award for Great Acedemic Achievements.

WORK/ADDITIONAL EDUCATION

2008-2012 **Belozersky Institute of Physico-Chemical Biology, Laboratory of Molecular Gene Biology** (Moscow, Russia). PI: Dr. Andrey Vartapetian.
Researcher
Conducted research on *Agrobacterium* VirD2 protein, its functions and interactions during plant programmed cell death.

- 2009: Creation of plasmid constructions expressing VirD2 C-terminal fragments *in planta.*
- 2010: Effects of VirD2 C-terminal fragments expression on plant cells.
- 2010: Full-size VirD2 protein as a substrate for phytaspase, a plant caspase-like protease.
- 2011: TATA-binding protein as a substrate for phytaspase, a plant caspase-like protease.
- 2012: VirD2 autoubiquitination.

2010: **Institute of Medical Biology, Vertebrate Development Signaling lab**.
Agency for Science, Technology and Research (A*STAR; Singapore, Singapore).
PI: Professor Michael Jones, Ph. D.
Research officer
Studied Arkadia E3 ligase protein and its roles during different stages of *Danio rerio* development, performed bioinformatical analysis of Arkadia protein.

2006: **Johns Hopkins University Center for Talented Youth (CTY).**
Medical Sciences: Pharmacology and Toxicology (Bethlehem, PA, U. S. A.).

2005: **Johns Hopkins University Center for Talented Youth (CTY).**
Biotechnology (Bethlehem, PA, U. S. A.).

AWARDS
2012: Full governmental scholarship to attend **M. V. Lomonosov Moscow State University** (Moscow, Russia).

2007: Recipient of four Diplomas of **Lomonosov Olympiad** foir *Biology*, *Chemistry*, *Mathematics* and *Russian Language/Literature* (an Olympiad recognizing outstanding university applicants).

2006, 2007: **Johns Hopkins University Center for Talented Youth (CTY)** diplomas for great performance.

VOLUNTEERING
2013: **Johns Hopkins Hospital** (Sheikh Zayed Building), Adult Emergency Department.

TEACHING
2013-2014: **Johns Hopkins Bloomberg School of Public Health**, Department of Biochemistry and Molecular Biology, Teaching Assistant for Biochemistry II course for Master's students. Professor: Floyd Randy Bryant, Ph.D